Dysfunction of the Retinal Pigment Epithelium with Age: Increased Iron Decreases Phagocytosis and Lysosomal Activity

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PURPOSE. Iron accumulation with age in the retinal pigment epithelium (RPE) may be one important source of oxidative stress that contributes to age-related macular degeneration (AMD). Young and old rodent RPE/choroid were compared to assess iron homeostasis during normal aging and the effects of increased iron on the functions of retinal pigment epithelial cells.

METHODS. The iron level, mRNA expression, and protein level of iron-regulatory molecules in RPE/choroid were quantitatively compared between young and old animals. To test the effects of increased intracellular iron on the functions of retinal pigment epithelial cells, in vitro ARPE-19 cells were treated with high levels of iron and assessed for phagocytosis activity and lysosomal activity.

RESULTS. Iron level was significantly increased in the aged RPE/choroid. Ferritin and ceruloplasmin mRNAs were significantly increased in the aged RPE/choroid, whereas transferrin, transferrin receptor, and ferroportin mRNAs did not change with age. At the protein level, decreased transferrin and transferrin receptor, increased ferritin and ceruloplasmin, and unchanged ferroportin were observed in the aged RPE/choroid. Exposure of ARPE-19 cells to increased iron markedly decreased phagocytosis activity, interrupted cathepsin D processing, and reduced cathepsin D activity in retinal pigment epithelial cells.

CONCLUSIONS. The RPE/choroid of aged animals demonstrates iron accumulation and associated alterations in iron homeostasis. Iron accumulation with age may impair the phagocytosis and lysosomal functions of retinal pigment epithelial cells in the aged RPE/choroid. Therefore, age-related changes of iron homeostasis in the RPE could increase the susceptibility of the tissue to genetic mutations associated with AMD. (*Invest Ophthalmol Vis Sci.* 2009;50:1895–1902) DOI:10.1167/iovs.08-2850

A ge-related macular degeneration (AMD), the loss of macular function from the degenerative changes of aging, is the leading cause of loss of central vision in the elderly in developed countries.[1,2] AMD is likely to be a multifactorial disease, caused by genetic, environmental, and tissue factors. Age as a risk factor for AMD may be related to functional changes in the neural retina, retinal pigment epithelium (RPE), and the choroid as these tissues age. The concept that AMD occurs on a background of predisposing, age-related tissue changes must be investigated further.[3]

Of many theories, increasing evidence shows oxidative stress and free radical damage in the RPE may underlie the pathogenesis of AMD.[4,5] The RPE generates reactive oxygen species through the phagocytosis of photoreceptor outer segment (POS), the digestion of phagocytosed POS,[6] and exposure to intense light.[7] Retinal pigment epithelial cells in the macular area, which has the highest oxygen consumption in the retina, carry an additional oxidative stress burden. Although many studies suggest that cumulative oxidative stress contributes to the pathogenesis of AMD, the sources of free radicals with age are not completely known.

Recent studies show iron may contribute to the pathogenesis of AMD as a source of free radicals that damage the tissues.[8,9] Iron is an essential element for many metabolic processes,[10] but excess iron generates highly reactive hydroxyl radicals that damage lipid membranes and proteins.[11] Changes of iron homeostasis have been associated with photoreceptor degeneration, such as in RCS (Royal College of Surgeons) rats[12] and AMD.[8,13] Excess iron has been observed in the macular region of the retina, in the RPE, and in the drusen of AMD-affected eyes.[14,15] A mouse model deficient in both ceruloplasmin (Cp) and hephaestin shows age-dependent retinal iron accumulation and retinal degeneration with AMD features.[16,17] Several studies, including ours, show iron levels increase normally with age in the neural retina.[18–20] However, there are few studies on iron levels in the RPE, the effects of aging, the physiological effects of excess iron on RPE function, and the way iron homeostasis in the RPE changes with age.

We hypothesize that changes in iron homeostasis in the aged RPE impair the functions of the RPE layer and predispose the tissue to a disease such as AMD. We studied young and old mice and rats to characterize changes in the state of iron homeostasis in the aged RPE/choroid. In addition, we present evidence that increased iron leads to impaired phagocytic and lysosomal functions of retinal pigment epithelial cells.

MATERIALS AND METHODS

Animals

All experimental protocols were in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. C57BL/6 male mice 4 and 26 months of age and Brown Norway male rats 4 and 30 months of age (National Institute on Aging, Bethesda, MD) were used in this study. We used mice for most of our work and rats for measurement of iron levels in the RPE/choroid because old mice were no longer available to us. All animals were housed under standard conditions and 12-hour cyclic light.

TABLE 1. Gene-Specific Primers Used in the Study

Gene	Forward Primer	Reverse Primer
Tf	gggatgacaccaaatgtttc	agcacttcctcatgttaccg
TrfR	ctaggaactggtgatccata	gagattgtttgcacaggtat
L-Ft	cagccgcctttacaagtctc	taggagctaaccgcgaagag
H-Ft	tgatgaagctgcagaaccag	gcacactccattgcattcag
Fpn	ctctgtcagcctgctgtttg	ggagttctgcacaccattga
Cp	ggccacagcttccaatacaa	gcatggacatggtcagtcac
18S	gtaacccgttgaaccccatt	ccatccaatcggtagtagcg

RPE/Choroid Dissection

Mouse and rat RPE/choroid were carefully dissected and completely separated from neural retinas and sclera. The mouse tissues were placed in RNA or protein lysis buffer. Two RPE/choroid from one mouse were pooled as one RNA sample. Four RPE/choroid from two mice were pooled as one protein sample. There were three samples from young animals and three from old animals.

Quantitative RPE/Choroid Iron and Manganese Detection

Iron levels in the RPE/choroid from three young and three old rats were detected by inductively coupled plasma-optical emission spectrophotometry (Vista-MPX ICP-OES; Varian Inc., Palo Alto, CA), as previously described.[21] Manganese levels in each sample were measured at the same time as an internal control. Standards were prepared from atomic absorption standard solutions of iron and manganese (1 g/L; Thermo Fisher Scientific, Waltham, MA). Standards, blanks, and standard curves were used as previously specified.[21] Serum iron of the same rat was detected (QuantiChrom Iron Assay Kit; Bioassay Systems, Hayward, CA) as another internal control.

Iron Treatment of ARPE-19 Cells

Human ARPE-19 cells were purchased from American Type Culture Collection (Manassas, VA) and maintained in DMEM/F12 with 10% fetal bovine serum. Cells at complete confluence were used for experiments. Iron was added into media as $FeCl_3$-sodium nitrilotriacetate (Fe-NTA) at final concentrations of 200 to 3000 μM[22] for 24 hours, and cell viability was quantitated by MTT assay (Promega, Madison, WI). Nontoxic doses of 500 μM, 1000 μM, and 2000 μM (see Fig. 4A) were used for the experiments.

Phagocytosis Activity Assay

POS was isolated from fresh bovine eyes obtained from the slaughterhouse, as described previously.[23] Isolated POS aliquots were stored at $-80°$C at a concentration of 10^9 POS/mL. Before use, POS was labeled with 1 mg/mL fluorescein isothiocyanate (FITC; Molecular Probes, Carlsbad, CA) in 0.1 M Na-bicarbonate (pH 9.0) for 1 hour in the dark before it was washed and resuspended in cell culture medium with 2.5% sucrose. Retinal pigment epithelial cells were fed with FITC-POS

(10 POS/retinal pigment epithelial cell) for 1 to 6 hours under culture conditions before they were rinsed three times with PBS containing 1 mM $MgCl_2$ and 0.2 mM $CaCl_2$. Total fluorescence was recorded at 485/525 nm with a microplate reader (Tecan; Männedorf, Switzerland) and was normalized to total protein assayed. Each assay was repeated three times.

Real-time RT-PCR

Total RNA was extracted from mouse RPE/choroid or ARPE-19 cells (RNeasy Protect Mini Kit; Qiagen, Valencia, CA). The concentration of RNA isolated was quantitated by spectrophotometry. Two hundred nanograms of RNA from each sample was reverse transcribed into cDNA (iScript cDNA Synthesis Kit; Bio-Rad Laboratories Inc., Hercules, CA) and was used for real-time PCR reactions. Specific primers (Table 1) were synthesized by Integrated DNA Technologies (Coralville, IA). PCR reactions were performed with detection software (iCycler; Bio-Rad) in triplicate. All data were collected from the linear range of the amplification. Expression levels were normalized to the 18S mRNA levels from the same samples.

Western Blot Analysis

Mice RPE/choroid or ARPE-19 cells were lysed in the extraction buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 1 mM EDTA plus protease inhibitors (Pierce, Germany) and were sonicated for 5 seconds before protein concentration determination by the Bradford colorimetric assay. Fifteen micrograms of proteins were separated on SDS-PAGE and electrotransferred onto polyvinylidene difluoride membranes. The membrane was blocked in 5% nonfat milk and 0.05% Tween 20 in Tris-buffered saline (TBS) and then was incubated with primary antibodies (Table 2) at $4°$C overnight. The membrane was rinsed with 0.05% Tween 20 in TBS and incubated with secondary antibody for 1 hour. Blots were developed by enhanced chemiluminescence (ECL). Relative band density was determined with ImageJ software (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at http://rsb.info.nih.gov/ij/index.html). β-Actin was used as loading and quality control.

Immunofluorescence Labeling

Eyes were fixed in 4% paraformaldehyde in PBS for 4 hours and embedded in paraffin. Five-micrometer–thick sections were dewaxed and rehydrated. For antigen retrieval, the sections were heated in 10 mM sodium citrate buffer (pH 6.0) at a sub-boiling temperature for 10 minutes, followed by cooling for 30 minutes. ARPE-19 cells were fixed in 4% paraformaldehyde in PBS for 10 minutes. Tissue sections or coverslips were incubated with primary antibodies (Table 2) diluted in 5% BSA in PBS overnight at $4°$C. Sections incubated with 5% BSA in PBS without primary antibody were used as negative controls. After several washes, samples were incubated with the secondary antibody. After washing with PBS, slides were mounted with medium containing DAPI (Vectashield; Vector Laboratories, Burlingame, CA). Staining was per-

TABLE 2. Primary Antibodies Used in the Study

Antibody	Source	Type of Antibody	Dilution (WB)	Dilution (IF)
Tf	Santa Cruz	Rabbit polyclonal	1:800	1:200
TrfR	Zymed	Mouse monoclonal	1:10,000	1:200
Ft	Sigma	Rabbit polyclonal	1:2000	1:800
Fpn	Orbigen	Rabbit polyclonal	1:500	1:400
Cp	BD Biosciences	Mouse monoclonal	1:500	1:200
Cat D	GeneTex	Rabbit polyclonal	1:10,000	—
ZO-1	Zymed	Rabbit polyclonal	—	1:200
β-actin	Sigma	Mouse monoclonal	1:20,000	—

WB, Western blot; IF, immunofluorescence labeling; Tf, transferrin; TrfR, transferrin receptor; Ft, ferritin; Fpn, ferroportin; Cp, ceruloplasmin; Cat D, cathepsin D.

FIGURE 1. Changes of iron level in the RPE/choroid of young and old rats measured by ICP-OES analysis. (**A**) Iron levels were significantly increased in the RPE/choroid of old rats ($P < 0.05$; $n = 3$). (**B**) Manganese levels in the RPE/choroid of young and old rats were not significantly different ($P > 0.05$; $n = 3$). (**C**) Serum iron of the young and old rats were not significantly different ($P > 0.05$; $n = 3$).
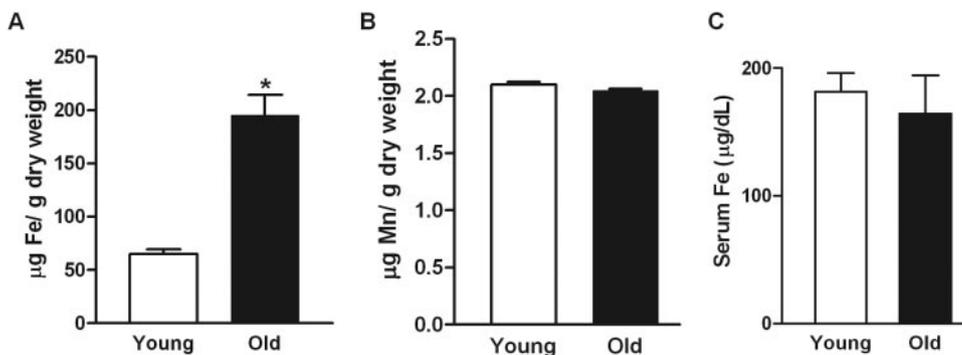
formed on four mice in each age group and was repeated three times for each antibody and each age. The results were consistent.

Lysosomal Enzyme Activities In Vitro

Cathepsin D activity was measured in retinal pigment epithelial cell extracts with the use of a kit containing a fluorogenic peptide substrate peptide, MOCAc-Gly-Lys-Pro-Ile-Leu-Phe-Phe-Arg-Leu-Lys(DNP)-D-Arg-NH2 (Sigma), reaction buffer (pH 4.0), and standards. Reactions were initiated by the addition of substrate, and the kinetics of substrate hydrolysis was measured at 340/460 nm using a fluorescent plate reader at 37°C for 15 minutes with data points collected every 120 seconds Each assay was repeated three times. Data were imported for analysis (Prism; GraphPad Software Inc., San Diego, CA) and determination of initial rates, and normalization to total protein was assayed.

Live Cell Lysosomal Enzyme Assay

The targeted peptide substrate (R9-Cat D) was developed by Fischer et al.[24] After iron treatment, cells were washed and incubated with serum- and dye-free culture media containing 2 μM R9-Cat D peptide for 1 hour. Media were removed, and cells washed twice with PBS. Then fluorescein (485/525) and rhodamine fluorescence (580/620) was sequentially recorded using a microplate reader (Tecan). Each assay was repeated three times.

RESULTS

Increased Iron Levels in Aged RPE/Choroid

ICP-OES results showed that the iron levels were significantly increased by threefold in the RPE/choroid of old rats (195.4 ± 33 μg/g [dry weight]) compared with the RPE/choroid of young rats (65.0 ± 8 μg/g; Fig. 1A). As an internal control, we also measured manganese levels in the same samples. Manganese levels in the young and aged RPE/choroid were 2.1 ± 0.04 μg/g and 2.0 ± 0.03 μg/g, respectively, and were not significantly different (Fig. 1B). To determine systemic iron levels, we measured the serum iron. There was no significant difference between young and old rats (181.6 ± 25.3 μg/dL and 164.4 ± 51.7 μg/dL, respectively; Fig. 1C). Thus, there was a significant increase in the tissue levels of iron in the RPE/choroid of old rats.

Changes in Iron-Regulatory Molecules in Aged RPE/Choroid

To maintain iron homeostasis locally in tissues, a series of iron-regulatory proteins tightly control cellular iron uptake, storage, intracellular distribution, and export. The main iron-regulatory molecules include transferrin (Tf), transferrin receptor (TrfR), ferritin (Ft), ferroportin (Fpn), and ceruloplasmin (Cp). Tf is the major iron transport protein. TrfR is the cell surface receptor for Tf, and the binding of Tf to TrfR enables the cells to take up iron by endocytosis.[25] Intracellular iron is

stored mainly in Ft,[25] which is composed of 24 subunits of ferritin light chain (L-Ft) and ferritin heavy chain (H-Ft). Fpn is the only putative iron exporter identified to date,[26] and Cp functions as a ferroxidase participating in the release of iron from cells.[27] We compared the gene expressions, protein levels, and cellular locations of these iron-regulatory molecules in the RPE/choroid of young and old mice by real time RT-PCR, Western blot, and immunofluorescence staining.

Real-time RT-PCR showed the gene expression levels of *L-Ft, H-Ft,* and *Cp* were significantly increased in the aged RPE/choroid ($P < 0.05$; Fig. 2), but *Tf, TrfR,* and *Fpn* mRNA expression did not show significant differences between young and old mice ($P > 0.05$; Fig. 2).

Although *Tf* and *TrfR* mRNA were not changed with age, the amount of protein synthesized from both *Tf* and *TrfR* were significantly decreased in the aged RPE/choroid, suggesting posttranscriptional downregulation of Tf and TrfR protein synthesis in the old mice ($P < 0.05$; Figs. 3A, 3B). The protein levels of Ft and Cp were significantly increased in the aged RPE/choroid ($P < 0.05$; Figs. 3A, 3B) and were consistent with the changes in mRNA level. There was no significant change in Fpn protein level in aged RPE/choroid ($P > 0.05$; Figs. 3A, 3B).

Immunofluorescence labeling showed all these iron-regulatory proteins were present in retinal pigment epithelial cells in young and old mice. Figures 3E and 3F show the labeling for Ft in retinal pigment epithelial cells in young and old mice. In the young eye, slight labeling for Ft is present in the RPE. In the old eye, dense particulate labeling for Ft can be seen throughout the RPE layer and in endothelial cells of the choroid, near the
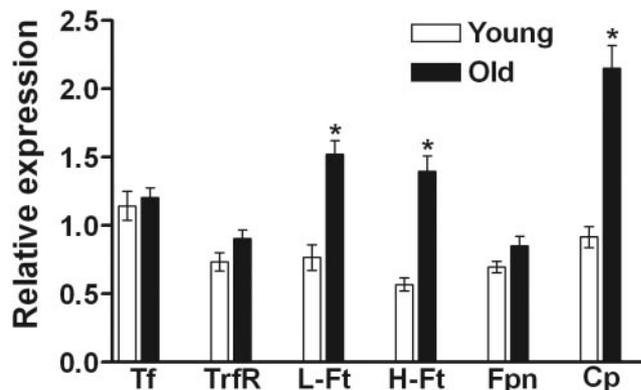

FIGURE 2. Gene expression of iron-regulatory molecules in the RPE/choroid of young and old mice measured by real time RT-PCR. mRNA expression levels of *L-Ft, H-Ft,* and *Cp* are significantly increased in the RPE/choroid of old mice (*$P < 0.05$; $n = 3$), but *Tf, TrfR,* and *Fpn* mRNA expression does not show significant differences between young and old mice ($P > 0.05$; $n = 3$). Tf, transferrin; TrfR, transferrin receptor; L-Ft, ferritin light chain; H-Ft, ferritin heavy chain; Fpn, ferroportin; Cp, ceruloplasmin.
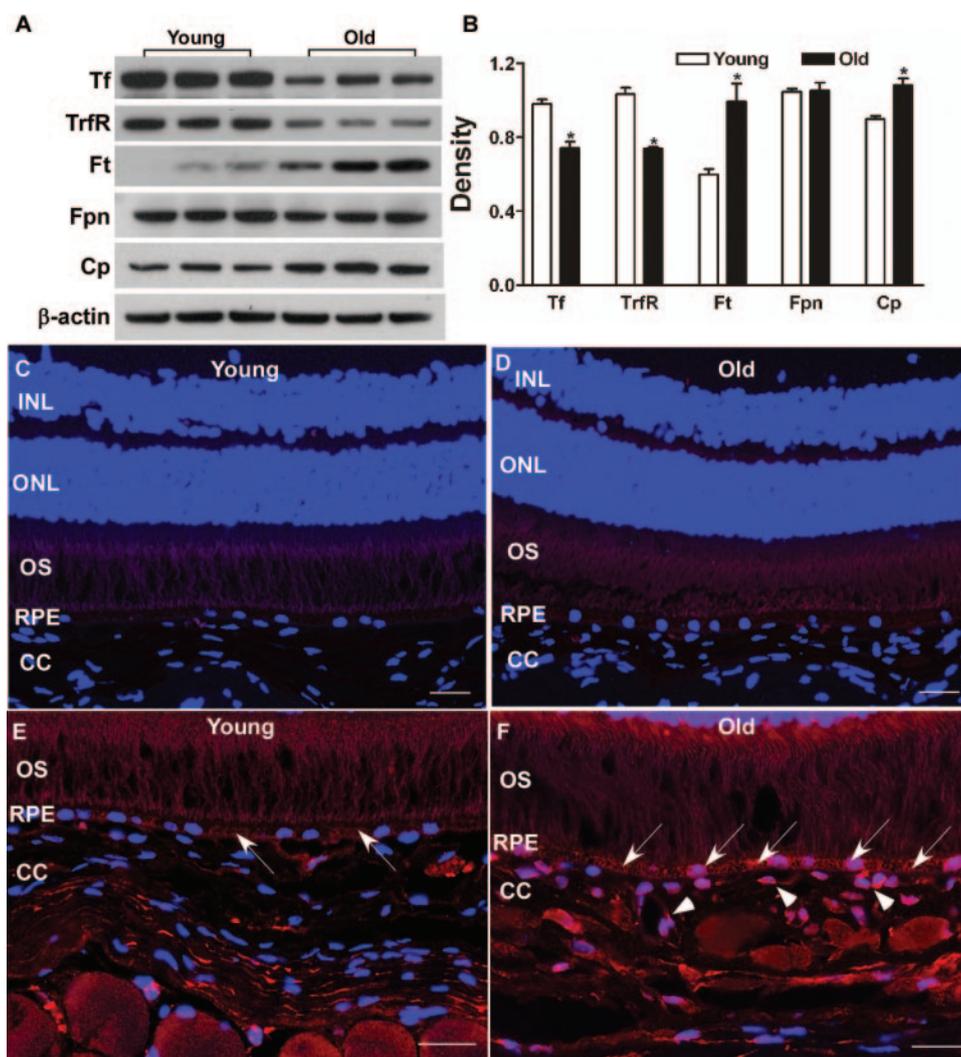
FIGURE 3. Protein levels and cellular localization of iron-regulatory molecules in the RPE/choroid by Western blot and immunofluorescence labeling. (A) Western blot shows the protein levels of Tf, TrfR, Ft, Fpn, and Cp in the RPE/choroid of young and old mice. Tf and TrfR protein levels are decreased in aged RPE/choroid, Ft and Cp protein levels are increased in aged RPE/choroid, and Fpn does not change. (B) Densitometric measurements confirm the changes in aged RPE/choroid (*$P < 0.05$; $n = 3$). (C–F) Representative images show the cellular localization of Ft in the RPE/choroid of young (E) and old (F) mice by immunofluorescence labeling. (C, D) There is no staining in the negative control slides, in which primary antibody was omitted. (E, *arrows*) There is slight Ft staining in the RPE of young eyes. (F) In the RPE of old mice, there is intense particulate staining throughout the RPE layer (*arrows*) and in the endothelial cells of choroid capillaries (*arrowheads*). The intensity of Ft staining in aged RPE/choroid was higher than that in the young RPE/choroid, consistent with the Western blot result. Scale bar, 25 μm. INL, inner nuclear layer; ONL, outer nuclear layer; OS, outer segment; RPE, retinal pigment epithelium; CC, choroid capillaries.

Bruch membrane. Other molecules showed similar cellular localizations (data not shown).

Decrease in the Phagocytosis Activity of RPE Cells Due to Elevated Intracellular Iron

Our results showed increased iron in the RPE/choroid of aged rats compared with that of young rats. This age-related, increased intracellular iron may cause dysfunction of retinal pigment epithelial cells. With the use of a human retinal pigment epithelial cell line, ARPE-19, we sought to determine whether increased intracellular iron would alter the physiological functions of RPE.

First, we determined in vitro the sublethal concentrations of Fe-NTA that would not cause the death of ARPE-19 cells. NTA is an iron carrier, and Fe-NTA enters cells rapidly and independently of TrfR-mediated iron uptake. Increased Fe-NTA concentrations in the culture media increase the intracellular iron level.[28] We found that increasing Fe-NTA levels up to 2000 μM in the media did not cause a significant decrease in cell viability ($P > 0.05$; Fig. 4A). Only 3000 μM Fe-NTA had a small cytotoxic effect on the ARPE-19 cells ($P < 0.05$). Therefore, we chose the concentrations of 500 μM, 1000 μM, and 2000 μM Fe-NTA (Fig. 4A, *arrows*) for further experiments.

We detected changes in the two major iron-regulatory proteins, TrfR and Ft, under increased iron conditions in vitro. Western blot showed the increased iron in vitro downregu-

lated TrfR and upregulated Ft proteins (Fig. 4B), consistent with the physiological response of retinal pigment epithelial cells to increased intracellular iron and with the age-related changes in the presence of excess iron in the aged RPE/choroid (Fig. 3A). We suggest that this model of iron loading mimics, at least in part, the intracellular iron accumulation and altered iron homeostasis that occurs with age.

Phagocytosis of POS is one of the most important functions of retinal pigment epithelial cells. Impaired phagocytosis function of RPE has been associated with the pathogenesis of AMD. To test the effect of increased iron on the phagocytic activity of retinal pigment epithelial cells, we performed phagocytosis assays that quantified the uptake of fluorescein-labeled POS (FITC-POS) by ARPE-19. Exposure to FITC-POS demonstrated continuous increased uptake of POS by ARPE-19 for 6 hours under control conditions. However, preincubating the cells in media containing a nontoxic concentration of 1000 μM Fe-NTA drastically lowered the rate of uptake of FITC-POS at all time points (Fig. 4C). We found that the effects of elevated iron on the phagocytosis activity of retinal pigment epithelial cells were concentration dependent. With pretreatment of 500 μM, 1000 μM, and 2000 μM Fe-NTA to the ARPE-19 cells, POS uptake was decreased by approximately 39%, 45%, and 78% of the total uptake, respectively, at 3 hours (Fig. 4D). Immunofluorescence labeling further confirmed that there was less POS uptake in the iron-treated cells than in the nontreated cells (Figs. 4E, 4F).

FIGURE 4. Elevated iron level decreases the phagocytosis activity of retinal pigment epithelial cells in vitro. Cell viability was measured through the MTT assay and was expressed as a percentage of viability compared with control. (**A**) Effect of Fe-NTA on the viability of ARPE-19 cells. Fe-NTA less than or equal to 2000 μM in the media does not cause a significant decrease in cell viability ($P > 0.05$). 3000 μM Fe-NTA in the media has a small cytotoxic effect on RGC-5 cells (*$P < 0.05$). Concentrations of 500 μM, 1000 μM, and 2000 μM Fe-NTA in the media were chosen for further experiments (*arrows*). (**B**) Western blot shows the responses of ARPE-19 cells to the elevated iron level, which induces the decrease of TrfR and the increase of Ft at a dose-response pattern. (**C**, **D**) The effect of elevated iron on the phagocytosis activity of retinal pigment epithelial cells. Phagocytosis activity is expressed as a percentage of the activity of control retinal pigment epithelial cells at 6 hours. (**C**) From 1 to 6 hours, 1000 μM Fe-NTA significantly inhibits the uptake of POS by retinal pigment epithelial cells. (**D**) The inhibitory effect of iron on the phagocytosis activity shows a dose-response pattern. (**E**, **F**) Immunofluorescence labeling shows the uptake of POS by retinal pigment epithelial cells after 3 hours. *Red*: ZO-1 demonstrating the tight junctions between the retinal pigment epithelial cells (*arrowhead*). *Green*: FITC-POS demonstrating the POS on the membrane and in the cytoplasm (*arrows*). The amount of POS uptake is decreased in the Fe-NTA–treated cells compared with the control cells. Scale bar, 50 μm. Fe500, 500 μM Fe-NTA in the media; Fe1000, 1000 μM Fe-NTA in the media; Fe2000, 2000 μM Fe-NTA in the media.
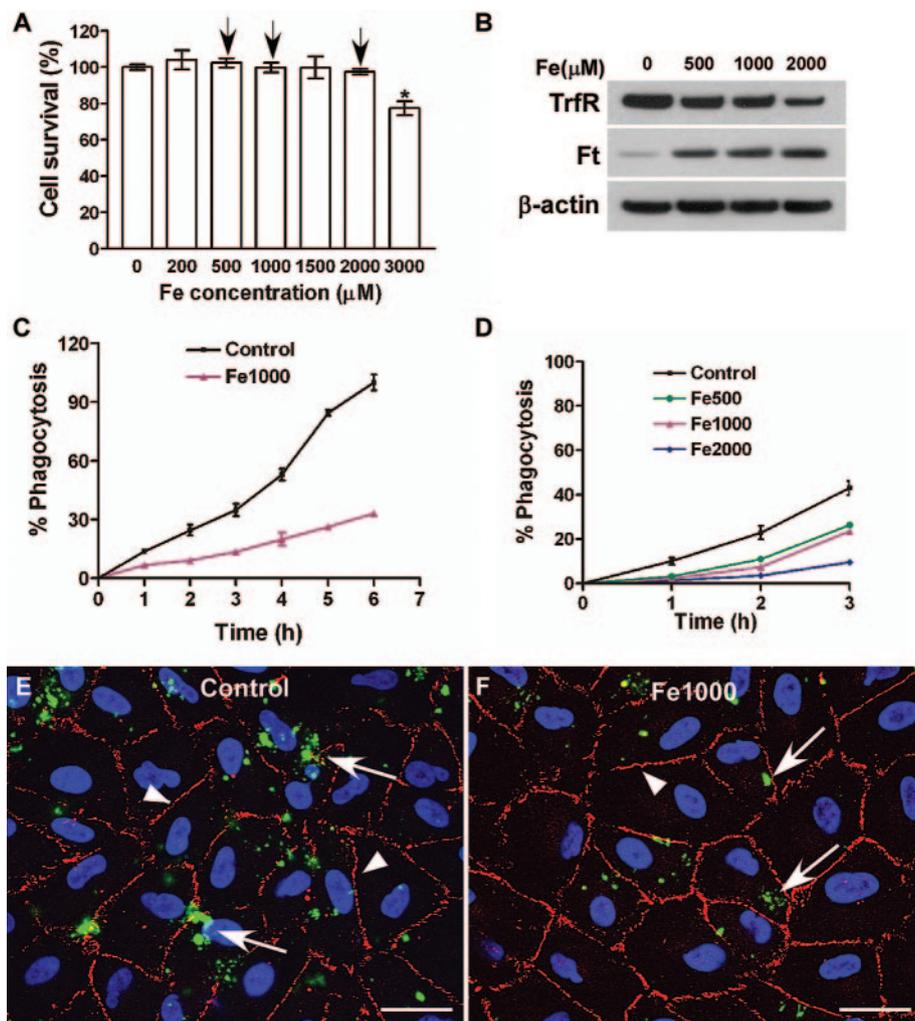
Decrease in the Lysosomal Activity of RPE Cells Due to Elevated Intracellular Iron

The key lysosomal enzyme involved in the lysosomal processing of POS is cathepsin D (Cat D).[29] Retinal pigment epithelial cells were treated with Fe-NTA for 24 hours and fed with POS for 3 hours, and then Cat D protein was measured. Western blot analysis showed there were two bands of Cat D. One band was the active form of mature Cat D at 34 kDa, and the other band was the inactive form of pro-Cat D at 48 to 52 kDa. Increased iron with or without exposure to POS caused a significant increase of pro-Cat D (Fig. 5A), quantified by densitometry (Fig. 5B).

Cat D is a protease that requires the acidic environment of the lysosome for its activity. Although the amount of mature Cat D protein was not visibly affected on the Western blot by iron, the activity of Cat D in the lysates from retinal pigment epithelial cells treated with 500 to 2000 μM Fe-NTA with or without POS was significantly decreased (Fig. 5C). POS alone did not affect Cat D activity in the lysate. To determine whether the decrease in Cat D activity measured in cell lysates could also be observed in live cells, we incubated the cells with a Cat D substrate peptide, R9-Cat D. The peptide has a fluorescein at the C terminus and a tetramethylrhodamine at the *n* terminus of the Cat D cleavage sequence. The peptide exhibits different fluorescence, depending on its cleavage status.[24] The red rhodamine fluorescence represents the amount of R9-Cat D inside the cells, and the green fluorescein fluorescence represents the amount of the C-terminal cleaved fragments. Therefore, the normalized ratio of green to red fluorescence represents the Cat D activity to cleave R9-Cat D in the lysosome compartments of the live cells. Our result showed that iron-treated cells with or without POS exhibited significantly decreased Cat D activity in live cells ($P < 0.01$; Fig. 5D). When R9-Cat D was used as a substrate in live cells, adding POS decreased the amount of fluorescence, most likely because of competition of R9-Cat D and protein from the POS for the enzyme.

DISCUSSION

Accumulation of redox-active iron in the retina is associated with several retinal degenerative diseases, including AMD, for which age is a major risk factor.[8,9] Thus, the changes of iron homeostasis with age may contribute to the underlying molecular basis of increased risk associated with age. RPE dysfunction is particularly relevant to the pathogenesis of AMD.[1] Here, we determined the changes of iron levels and the expression of iron-regulatory molecules in RPE/choroid that occur with nor-
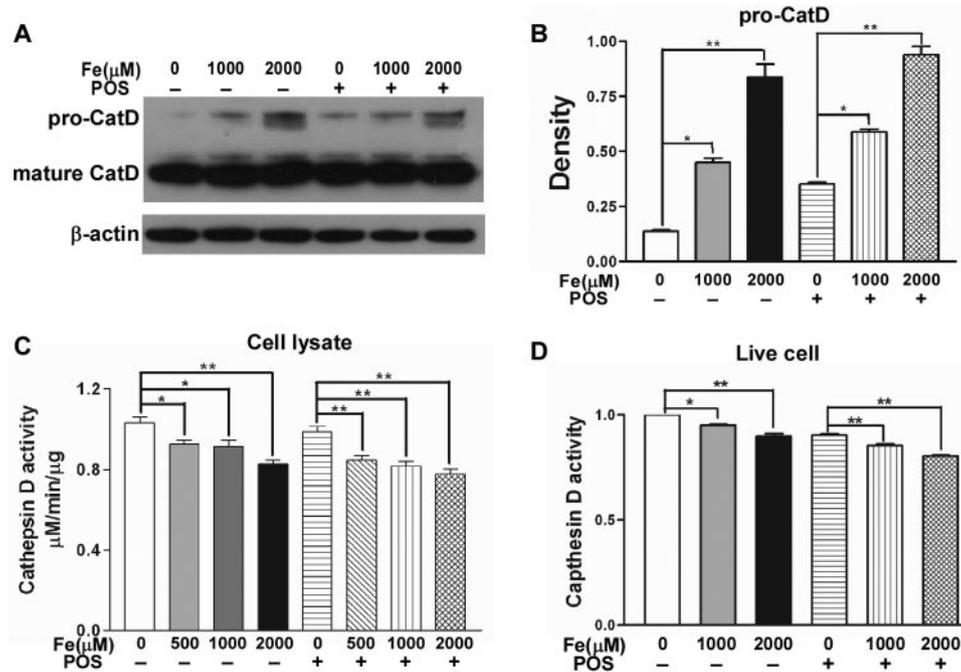
FIGURE 5.   Elevated iron level decreases the Cat D processing and activity of retinal pigment epithelial cells in vitro. (**A**) Western blot shows the effect of elevated iron level on the protein level of Cat D. Anti–Cat D antibody recognizes two bands of Cat D. One band, at 34 kDa, is the active form of mature Cat D, and the other band, at 48 to 52 kDa, is the intermediate form pro-Cat D. Increased iron with or without exposure to POS caused a significant increase of pro-Cat D. The amount of active Cat D shows no significant change. (**B**) Densitometric measurements confirm the significant difference shown by Western blot. (**C**) Cat D activity measurements in cell lysates from cells with different treatments. Retinal pigment epithelial cells treated with 500 μM, 1000 μM, and 2000 μM Fe-NTA, with or without POS, have significantly decreased Cat D activity. (**D**) Cat D activity measurement in live cells with different treatments. Retinal pigment epithelial cells treated with 1000 μM and 2000 μM Fe-NTA, with or without POS, significantly decreased the Cat D activity in the lysosome compartments of the live cells (*$P < 0.05$; **$P < 0.01$).

mal aging and assessed the effects of iron on several of the key physiological functions of retinal pigment epithelial cells in vitro. Our results suggest there are age-related changes of iron homeostasis in the normal, aged RPE/choroid, including accumulation of iron and associated changes of iron-regulatory molecules. Furthermore, at least in vitro and likely in vivo, elevated levels of intracellular free iron decrease the phagocytic and Cat D activities of retinal pigment epithelial cells.

Iron Accumulation in the RPE/Choroid with Age

Because iron is absorbed from the diet and little is excreted, iron accumulates and total body iron level increases with age. For example, iron accumulates in many regions of the brain with age,[30,31] and local mismanagement of iron homeostasis is associated with age-related neurodegeneration, such as Parkinson disease[32,33] and Alzheimer disease.[34] Age-related increases in iron level have been observed in the neural retinas of humans and rodents.[18–20] Previous work on human RPE/choroid demonstrated great variability of samples, and no clear conclusion was made.[19] Our study indeed showed an age-related accumulation of iron in RPE/choroid of rat that was independent of serum iron levels. These results were consistent with many other studies showing the lack of correlation between iron levels in local tissues and serum iron levels. Mice deficient in Cp and hephaestin are anemic, but abnormal iron accumulation is found in their retinas.[17] Patients with aceruloplasminemia are anemic but have retinal iron overload.[35] In our study, although the systemic iron level was within the

normal range in healthy aged animals, iron accumulation was increased in the aged RPE/choroid, suggesting a tissue-specific, iron-regulating mechanism.

We previously reported a modest but significant increase in iron level in the aged rat neural retina compared with the young rat neural retina.[18] Surprisingly, the difference in iron level in the RPE/choroid between young and old rats (3.0-fold) was higher than that in the neural retina (1.3-fold).[18] Iron is taken up at the basolateral surface of retinal pigment epithelial cells and is transported to the apical surfaces of retinal pigment epithelial cells, where it is released to the neural retina.[36] Therefore, iron flow to the neural retina may be limited and controlled by the RPE layer. The RPE, which maintains the outer blood-retinal barrier, may protect the neural retina from the effects of systemic iron overload or deficiency.

Iron level in the local tissue is tightly regulated by a group of iron-regulatory molecules. Iron is taken up by retinal pigment epithelial cells through Tf-mediated uptake after binding to TrfR. Intracellular iron is sequestered by Ft and stored as a nontoxic form. The iron that is not used or stored by the cell is exported by Fpn with the assistance of Cp.[25] Our study showed the significant decreases of Tf and TrfR proteins and the significant increases of Ft and Cp proteins in aged RPE/choroid. Together with a high level of iron in aged RPE/choroid, the changes of iron-regulatory molecules suggest that the retinal pigment epithelial cells from old animals sense an increased intracellular iron level and have a negative feedback mechanism to downregulate the synthesis of Tf and TrfR, minimize the free, toxic iron in the cytoplasm by increasing Ft

for iron storage, and convert toxic ferrous iron to nontoxic ferric iron by increasing Cp.

Changes in the iron-regulatory proteins TrfR and Ft are known to be regulated by a well-characterized regulatory system—iron-responsive elements (IREs)/iron-regulatory proteins (IRPs)[37]—that senses intracellular iron levels. In iron deficiency, binding of IRPs to the IRE inhibits the translation of Ft and protects TrfR mRNA from rapid degradation, thus leading to decreased Ft and increased TrfR. On the contrary, in iron overload, IRPs are inactivated or degraded, which results in increased translation of Ft and decreased translation of TrfR.[38] Our results, which showed increased synthesis of Ft and decreased synthesis of TrfR, suggest that the iron-regulatory molecules were posttranscriptionally regulated in the aged RPE/choroid, as they are under conditions of iron overload.

Our findings demonstrate that, in the RPE/choroid of rodents, as a normal, functional, age-related change, there is an accumulation of iron and an associated phenotypic alteration in iron homeostasis. Although these changes in iron homeostasis may not cause the death of retinal pigment epithelial cells, they may cause impairment of important physiological functions of retinal pigment epithelial cells.

Decrease of Physiological Activities of RPE Cells Due to Increased Intracellular Iron

Important functions of the RPE are phagocytosis of the shed tips of the POS and breakdown of the damaged photoreceptor proteins and lipids. However, the age-related accumulation of iron in RPE may impair these physiological functions. We used an in vitro retinal pigment epithelial cell line, ARPE-19, to determine the effects of sublethal iron accumulation on cellular functions. ARPE-19 is a human retinal pigment epithelial cell line that has been widely used to model retinal pigment epithelial cells for in vitro experiments. We increased intracellular free iron by using Fe-NTA in the media.[28,39–41] The two major iron-regulatory proteins, TrfR and Ft, showed the same responsive changes as those that occurred in vivo with age.

POS phagocytosis is one of the key functions of RPE to serve photoreceptors, and it relates RPE to retinal degenerations. Defects in POS phagocytosis promote photoreceptor degeneration, as in the RCS rat,[42] in *mer* knockout mice[43] and $\beta5$ knockout mice.[44] Our data show that elevated intracellular iron levels markedly decreased the rate of POS uptake by ARPE-19 cells, and the effects of iron overload were concentration dependent. Excessive iron can cause oxidative damage through the generation of reactive oxygen species by the Fenton reaction.[9,11] Sublethal oxidative stress by hydrogen peroxide inhibits RPE phagocytosis of POS through the activation of energy-sensing pathways.[45] Our finding that elevated intracellular iron dramatically decreased the POS phagocytosis of retinal pigment epithelial cells is consistent with the study on hydrogen peroxide and suggests that the effect of iron occurs likely through the generation of oxidative stress.

After retinal pigment epithelial cells phagocytose the tips of photoreceptors, they digest POS with the use of lysosomal enzymes and release the waste products into the choroidal circulation. Therefore, digestion capacity, represented by lysosomal activity, is another key function of retinal pigment epithelial cells. Cat D is the major lysosomal enzyme responsible for 80% of the degradation of rhodopsin.[29] It is an intracellular aspartic protease and has a complex maturation process that yields the active enzyme.[46] After pro-Cat D (48–52 kDa) is segregated into the acidic endolysosomal compartments, the pro-Cat D undergoes one or more proteolytic events, which may involve the cysteine proteases Cat L and Cat B,[47] to generate mature Cat D (34 kDa). Only mature Cat D is active in the lysosomes. Our results showed that iron overload increased

the amount of the inactive form of pro-Cat D, which suggested that excess iron inhibited the processing of pro-Cat D to the active form, Cat D, probably by the inhibition of cysteine protease or Cat B.[48,49] A previous study demonstrated that the presence of pro-Cat D is associated with increased accumulation of undigested POS-derived debris.[50] In addition, the enzyme activity of Cat D was significantly decreased by iron overload with and without POS as well as in cell lysates and in live cells.

In vitro, iron overload has a moderate effect to decrease Cat D activity and, presumably, lysosomal activity. Nevertheless, in vivo, iron overload over time, during aging, may lead to progressive accumulation of incompletely digested products of POS, such as lipofuscin, within the retinal pigment epithelial cells. An accumulation of lipofuscin occurs with age and is believed to be associated with the pathogenesis of AMD.[51] An association between decreased Cat D activity and AMD is also supported by an AMD mouse model, a transgenic mouse expressing a defective Cat D that develops features of AMD including lipofuscin accumulation and basal deposits.[52]

Retinal pigment epithelial cells serve the photoreceptors by phagocytosing and degrading POS daily throughout life without turnover of the retinal pigment epithelial cells themselves. Thus, even a small defect in this process could lead to considerable accumulation of undigested products with age. Our in vitro iron-loading studies suggest that the iron accumulation we observed in the normal RPE/choroid of old mice may impair key functions of retinal pigment epithelial cells, such as phagocytosis and lysosomal activity. Therefore, iron accumulation in aged RPE/choroid may predispose the RPE to decreased functions and degenerative changes.

In summary, our study suggests an age-related accumulation of iron and alteration of iron homeostasis in the rodent RPE/choroid. Whereas elevated intracellular iron is probably not the primary cause of AMD, the age-related changes that we report may compromise the functions of the RPE in old animals. To the extent that increased iron occurs in human RPE with age, iron overload may be an age-related susceptibility factor or risk factor that exacerbates the development of AMD. Therapeutic strategies to ameliorate age-related iron accumulation in retinal pigment epithelial cells may provide new treatments for age-related retinal diseases such as AMD.

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